# Factors Affecting Enantiomeric Fractions of Hexabromocyclododecane

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## Introduction

Hexabromocyclododecane (HBCD) is the most widely used of the cycloaliphatic brominated flame retardants (BFRs), and is an additive flame retardant used in polystyrene foams and upholstery textiles. Technical 1,2,5,6,9,10-HBCD is produced by bromination of cis, trans, trans cyclododecatriene (CDT); the resulting mixture contains three predominant diastereomers (α-, β-, and γ-HBCD, Becher 2005). These diastereoisomers exhibit markedly different physico/chemical properties (e.g., water solubility) that can influence rates of bioaccumulation, metabolism, and deputation. Recent reports provide evidence of bio-isomerization of the diastereoisomers in fish with a preferential formation of the α-isomer (Tomy et al. 2004, Gerecke et al. 2003, Allchin et al. 2003). The individual α-, β-, and γ-HBCD diastereoisomers are represented by a corresponding pair of enantiomers. Industrially synthesized chiral compounds are generally produced as racemates, and correspondingly released into the environment as racemates (Janak et al. 2005). Integrity of the racemic mixture is maintained when subjected to processes such as hydrolysis, photolysis, leaching, volatilization and atmospheric deposition. However, alterations in enantiomeric composition might occur as a result of biological processes such as metabolization (Janak et al. 2005).

Given the current interest in HBCD and its role as an environmental contaminant, an understanding of the environmental and biological fate of HBCD enantiomers is required. Janak et al. (2005) reported significant enantioselectivity for both α- and γ-HBCD in some biota samples from the Western Scheldt Estuary in Belgium using LC/MS/MS. We used similar methodology in the current investigation of enantiomer-specific accumulation of HBCD in a Lake Ontario food web using LC-MS-MS with electrospray ionization (ESI) in negative ion mode. We also previously reported that a number of factors influence accurate quantitation of HBCD diastereomers in complex environmental mixtures, including matrix effects and instrument response (Tomy et al. 2005). This current work also serves as an investigation of factors influencing the mass spectrometric response of the HBCD enantiomers, and therefore on enantiomeric fraction (EF) calculations.

## Materials and Methods

Lake Ontario pelagic food web samples (lake trout, alewife, rainbow smelt, slimy sculpin, mysids, and amphipods) were previously analyzed for HBCD diastereomers (Tomy et al. 2004); these same extracts were used for our study of HBCD enantiomers.

Fish (8-15 g wet weight) samples were extracted by accelerated solvent extraction, lipids were removed by gel permeation chromatography and the extracts were subjected to Florisil chromatography. HPLC was performed on an Agilent 1100 Series LC (Mississauga, ON) using a chiral column (4.0 x 200 mm, 5 µm) containing permethylated-cyclodextrin on silica -Nucleodex (Macherey-Nagel, Germany). Samples (2 µL injected) were analyzed on a MDS/Sciex 4000 QTrap hybrid triple quadrupole/linear ion trap mass spectrometer (Concord, ON) in electrospray ionization negative ion mode using multiple reaction monitoring (MRM) for the $[M-H]^-$ (m/z 640.6) → $Br^-$ (m/z 78.9 and 80.9) transition. The mobile phase consisted of water, methanol and acetonitrile at a constant flow rate of 500 µL per minute. Initial solvent conditions were 42% water/30% methanol/28% acetonitrile which was changed linearly over 14 minutes to a final solvent composition of 30% methanol/70% acetonitrile. Final conditions were held for 6 minutes before a linear ramp to the initial solvent conditions and equilibration for 30 minutes.

**Results and Discussion**

The chiral composition of HBCD was expressed as the enantiomeric fraction (EF), defined as $EF = A_+/(A_+ + A_-)$ where $A_+$ and $A_-$ are the first and second eluting enantiomer, respectively. Previous work identified the (-) α, (-) β and (+) γ HBCD as the first eluting peak of each corresponding enantiomeric pair (Heeb et al. 2005). A racemic compound in theory will have an EF = 0.5; any significant deviation from 0.5 indicates a shift in enantiomeric composition.

The analysis of HBCD standards containing the three diastereoisomers by ESI LC-MS-MS (Figure 1) resulted in EFs slightly lower than 0.5 for α- and β-isomers, but markedly higher than 0.5 for the γ-isomer. This effect was observed for a range of concentrations (20-200 pg on column), and was most pronounced for γ-HBCD at higher concentrations (Table 1). Inter-run variability of the EFs was greater at lower concentrations. Analysis of the $^{13}C$- and $d_{18}$- labeled HBCD diastereoisomers gave similar results. Given the HBCD standard mixture is racemic, variations in EFs from 0.5 were presumably due to differences in instrument response, with the potential additional influence of matrix effects in environmental samples. Also shown in Table 1 are "corrected" values, i.e., individual HBCD enantiomers were also quantified based on corresponding $d_{18}$-labelled analogues added prior to injection as instrument standards. Using corrected enantiomer concentrations, all HBCD EFs were racemic.

| | α-HBCD | α-HBCD | β-HBCD | β-HBCD | γ-HBCD | γ-HBCD |
|---|---|---|---|---|---|---|
| | Raw EF (N=7) | Corrected EF (N=7) | Raw EF (N=7) | Corrected EF (N=7) | Raw EF (n=7) | Corrected EF (N=7) |
| HBCD 200 pg | 0.48 ± 0.01 | 0.50 ± 0.02 | 0.47 ± 0.02 | 0.50 ± 0.04 | 0.62 ± 0.02 | 0.51 ± 0.02 |
| HBCD 50 pg | 0.50 ± 0.03 | 0.53 ± 0.03 | 0.51 ± 0.02 | 0.53 ± 0.04 | 0.56 ± 0.01 | 0.48 ± 0.01 |
| HBCD 20 pg | 0.49 ± 0.04 | 0.49 ± 0.04 | 0.49 ± 0.04 | 0.48 ± 0.05 | 0.55 ± 0.03 | 0.49 ± 0.03 |

**Table 1.** Enantiomeric fractions (EFs) for HBCD diastereoisomers in pure standards. [a]arithmetic mean +/ standard deviation.

| Sample | Raw EF | Corrected EF | Raw EF | Corrected EF |
|---|---|---|---|---|
| | α-HBCD | α-HBCD | γ-HBCD | γ-HBCD |
| Lake Trout #1 | 0.43 | 0.51 | 0.68 | 0.52 |
| Lake Trout #2 | 0.39 | 0.47 | 0.62 | 0.54 |
| Lake Trout #3 | 0.43 | 0.50 | 0.65 | 0.54 |
| Lake Trout #4 | 0.40 | 0.51 | 0.53 | 0.57 |

**Table 2.** Enantiomeric fractions (EFs) for HBCD in Lake Ontario lake trout.

Quantitation of HBCD diastereoisomers in the Lake Ontario food web samples were previously reported (Tomy et al. 2004). The α-isomer was consistently greater than the γ-isomer, while the β-isomer was below method detection in all samples (Figure 2). Corresponding EFs for selected Lake Ontario food web samples are shown in Table 2. Although the "uncorrected" profile shown in Figure 2 would suggest enantioselectivity for the Lake Ontario Lake Trout samples, i.e., a predominance of the (-) α- enantiomer over the (+) α-enantiomer, the corrected values in Table 2 show the true EFs are much closer to being racemic. This clearly highlights the need for using internal standards to benchmark the EF values. However, corrected EFs for the γ-HBCD enantiomers indicate the potential for enantioselectivity of these isomers, as the EF values were all substantially greater than 0.5.

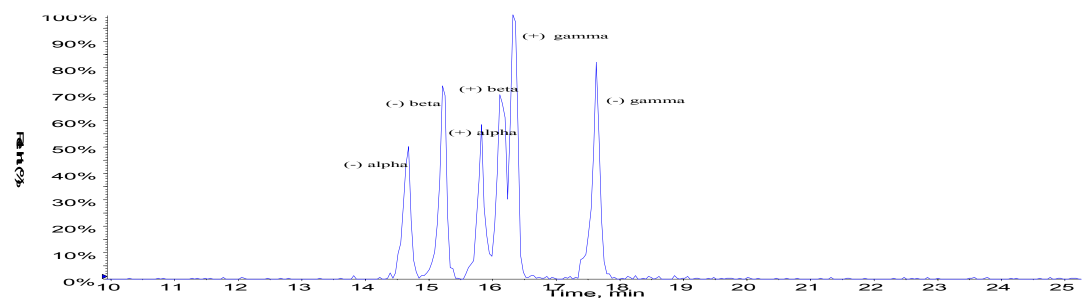


**Figure 1**. LC/MS/MS chromatogram of HBCD enantiomers in a standard mixture.

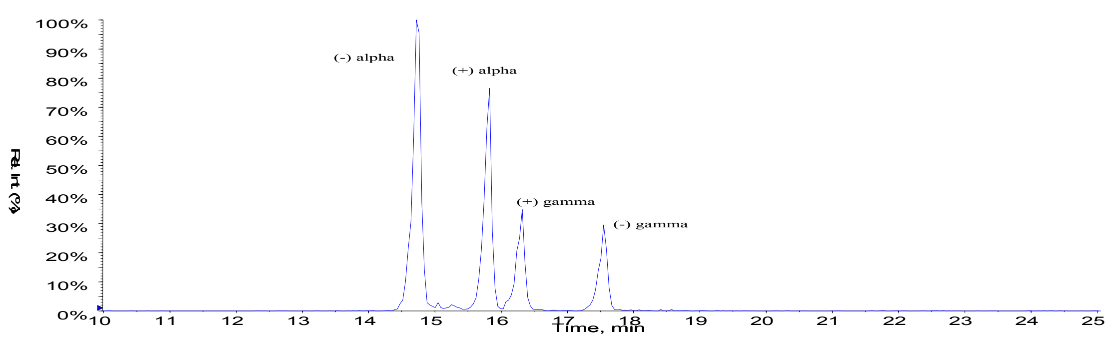


**Figure 2.** LC/MS/MS chromatogram of HBCD enantiomers in Lake Trout from Lake Ontario.

The reasons for the non-racemic EFs of HBCD standard solutions, and for the apparent differences in mass spectrometric response between individual enantiomers of the HBCD diastereomers, remain unclear. Our most recent work with new HPLC columns resulted in chromatograms and corresponding EFs for all 3 diastereomers that confirmed the racemic nature of the standards (Figure 3). These results indicated that some shifts in HBCD EFs may be due to chromatographic factors. We are currently investigating bleed from chiral columns as a potential factor in our observations.

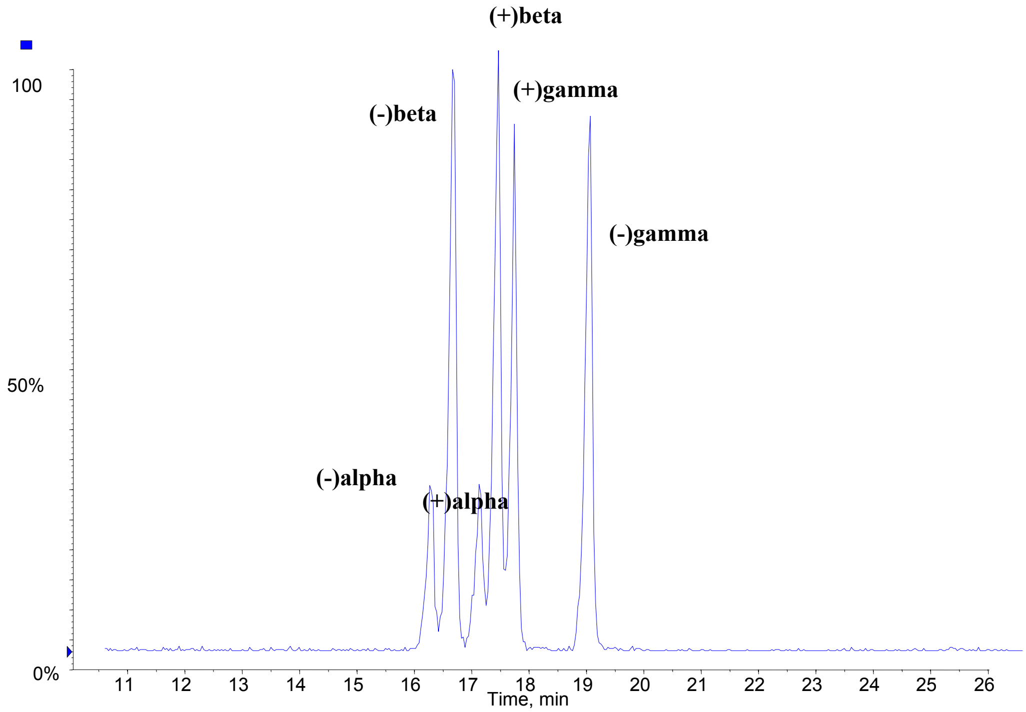


**Figure 3.** Enantiomeric separation of HBCD in standard mixture using a new column.

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